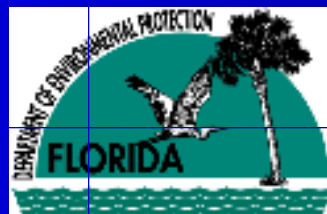# Performance Criteria and Field Testing of qPCR Methods for Human-Associated Microbial Source Tracking Markers and Fecal Indicator Bacteria in Fresh and Salt Surface Waters
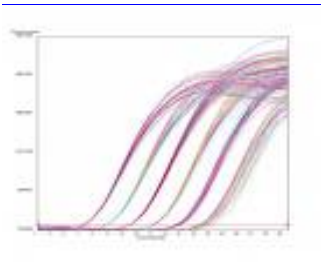
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EPA Beaches Conference 03/16/11

Study Objectives

3 Year Study:

- **Evaluate the performance of two qPCR-based MST methods for human sewage contamination**

- **Evaluate the performance of qPCR methods to quantify *E. coli* and enterococci**

- **Compare qPCR methods for *E. coli* and enterococci with culture (membrane filtration) methods**

- **Determine correlation between human markers and fecal indicator bacteria**

- **Inter-laboratory comparison of method transferability**

Literature Review Conducted to Select Two Human MST Markers

Criteria included:

- **Specificity**
- **Sensitivity**
- **Quantity in wastewater (limit of detection)**
- **Use in multiple labs**
- **Use across geographic regions**
- **Detection correlated with fecal indicator bacteria (FIB), pathogens, human health outcomes**

The picks: human *Bacteroides* HF183 and human polyomaviruses (HPyVs)

Other qPCR Targets
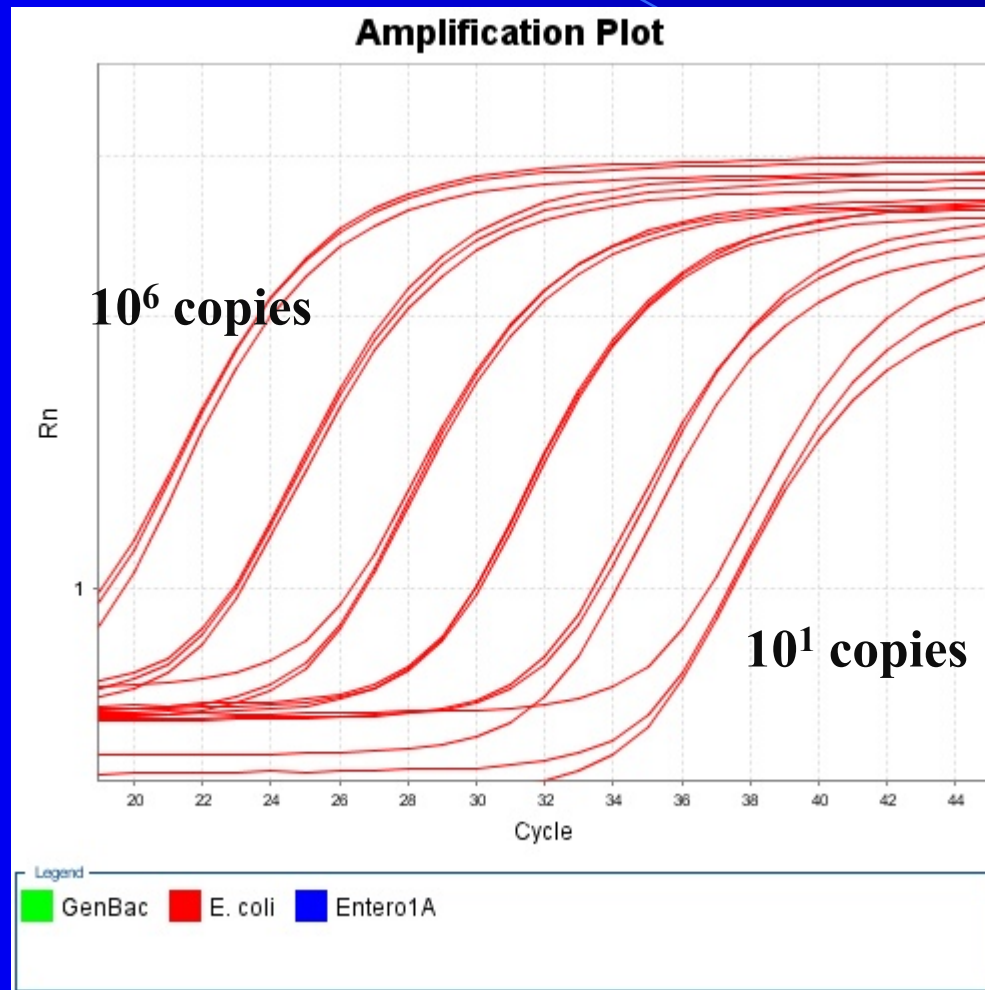# Fecal Indicator Bacteria

- *Enterococcus faecium* 23S rRNA (EPA; Ludwig & Schleifer, 2000)

- *E. coli uidA* gene (Lee 2005)

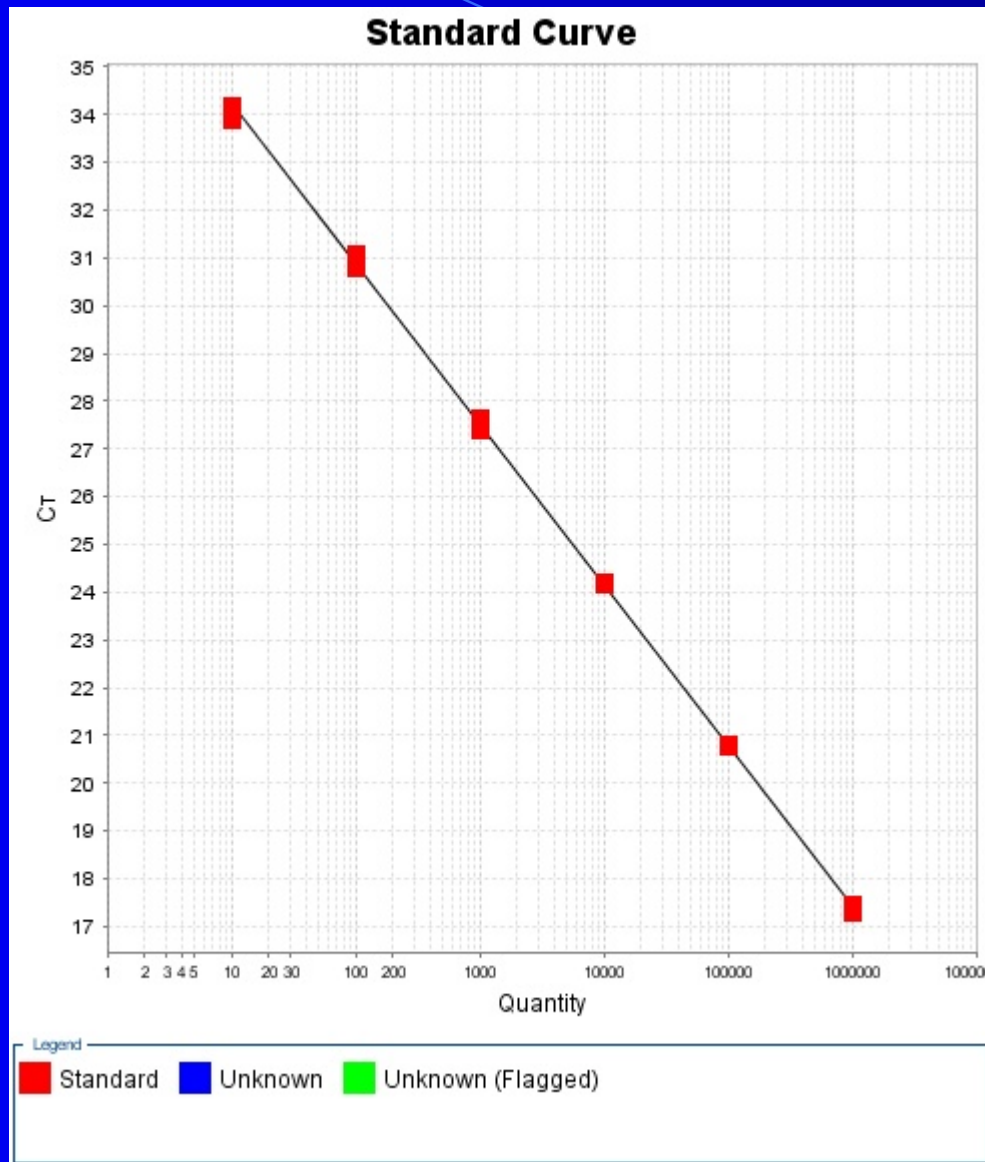- General *Bacteroidales* (EPA; Siefring et al., 2008)

E. coli qPCR Assay - Plasmid

E. coli Standard Curve

$r^2 = 0.9995$

Efficiency = 98.6

Standard deviation (C_T)
=0.063 for 10^6 copies
=0.325 for 10^1 copies

Standard Curve

Metrics

- **Sensitivity – ability to detect PCR marker when contamination from specific source is present**

- **Limit of detection – quantitative assessment of sensitivity, i.e. how little can we reliably detect?**

- **Specificity – PCR marker should not be detected when sewage from specific source is absent**

Method Details

- **Standards made from synthesized sequences (IDT)**
- **Standard curve run in triplicate reactions for each 96-well plate**
- **Samples and controls run in triplicate reactions**
- **Sensitivity (+/-) and method detection limit (quantitative) on sewage samples**
- **Specificity – non-targets include dogs, gulls, cattle**
- **Internal amplification control (IAC) multiplexed with general *Bacteroidales* assay**

Specificity

- **Tested against dog, cow, bird feces**
- **The HPyVs marker was 100% specific**
- **Human Bac HF183 was 81% specific**
- **HF183 cross-reacted with dog*, chicken and duck feces**
- ***detectable, but not quantifiable in dog feces**

Detection Limit for Sewage in Ambient Waters

- **Sewage spike 5 ml into 500 ml phosphate buffer → filter & extract DNA →dilution series ← qPCR**

- **Repeat procedure spiking sewage into lake, river, tannic, estuarine, marine waters**

- **Compare sample detection limit in buffer vs. ambient waters**

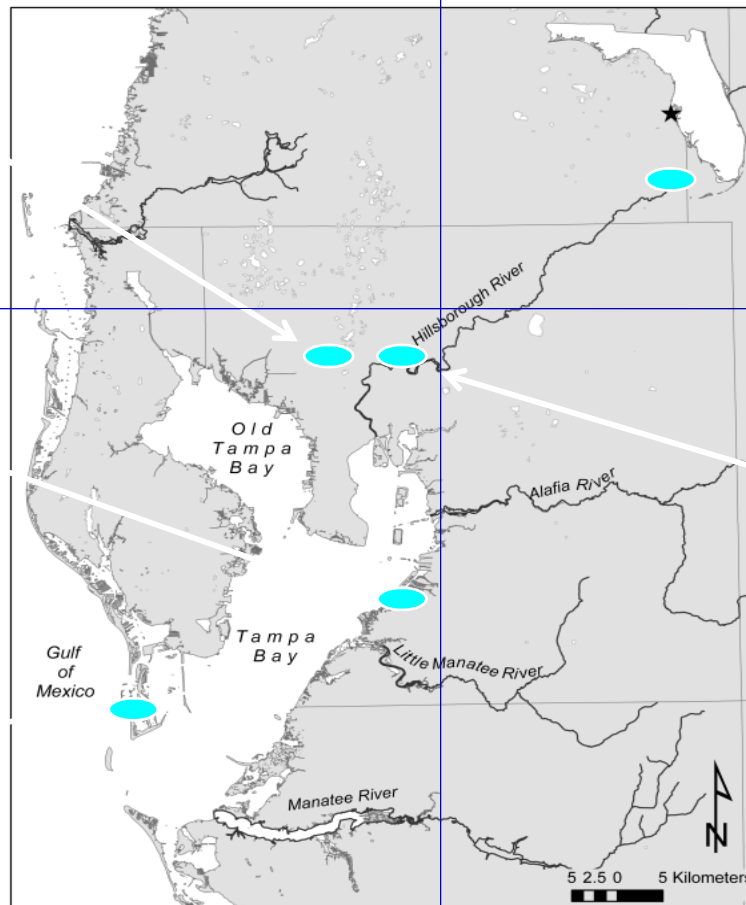- **Internal amplification control checks for inhibition**

Ambient Water Samples

Lake Carroll

Bahia Beach (Tampa Bay)

Fort DeSoto

Green Swamp

Riverfront Park Hillsborough River

Effect of Dilution of Sewage in Ambient Waters on MST Marker Quantification and Detection by qPCR

Site	HPyVs			HF183		
	Limit of Quantification		Limit of Detection	Limit of Quantification		Limit of Detection
	Target Copies/5µl[a]	Sewage Dilution[b]	Sewage Dilution	Target Copies/5µl[a]	Sewage Dilution[b]	Sewage Dilution
Bahia Beach	3.23×10^2	10^0	10^{-1}	8.31×10^2	10^{-2}	10^{-4}
	1.06×10^1	10^{-1}	10^{-1}	6.63×10^1	10^{-1}	10^{-1}
Fort DeSoto	2.05×10^1	10^{-2}	10^{-2}	8.81×10^1	10^{-3}	10^{-4}
	3.07×10^1	10^{-1}	10^{-1}	9.07×10^1	10^{-3}	10^{-3}
Green Swamp	3.91×10^2	10^0	10^{-1}	1.66×10^2	10^{-3}	10^{-3}
	1.64×10^2	10^0	10^0	1.31×10^1	10^{-3}	10^{-4}
Lake Carroll (site 6)	1.20×10^2	10^0	10^{-1}	1.98×10^2	10^{-3}	10^{-4}
	6.17×10^1	10^0	10^0	5.23×10^2	10^{-2}	10^{-2}
Hillsborough River[c]	5.47×10^1	10^{-2}	10^{-2}	1.71×10^1	10^{-4}	10^{-4}
	3.11×10^1	10^0	10^{-2}	4.30×10^2	10^{-2}	10^{-3}

PCR Inhibition in Ambient Waters Detected by Internal Amplification Control

Sample Site	C_T Value	
	Sampling Date 1	Sampling Date 2
Distilled water	35-38	
Bahia Beach	35.1	35.4
Fort DeSoto	36.4	35.6
Green Swamp	40.1	37.8
Lake Carroll	39.0	37.9
Hillsborough River	42.4	Undetermined

**Inhibition best relieved by template dilution**

Task 2: Persistence Study

Mimic persistence of markers in estuarine waters & beach following sewage spill
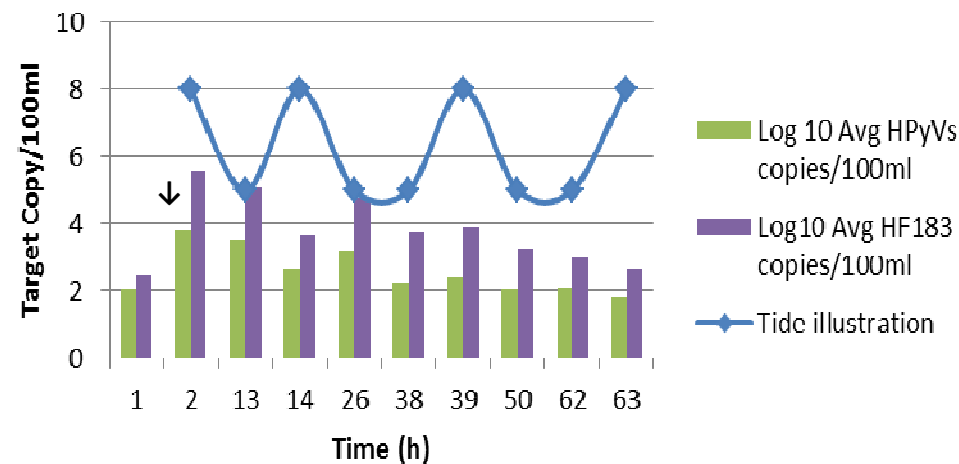
- **Sewage applied to sand**
- **Cycles of sand wetting and drying (approximating tidal cycles)**
- **Measure two MST markers by qPCR and FIB (enterococci & fecal coliforms) by membrane filtration in water**

Bacterial Log10 CFU/100ml vs time with Tide overlayed
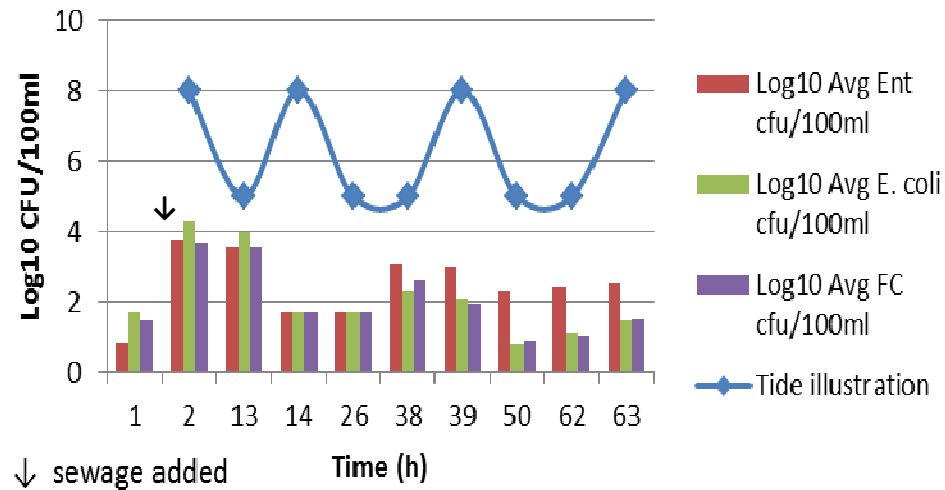
Log 10 Target Copy/100ml vs time with Tide overlayed

Correlation of Decay Rates of FIBs and MST Markers

		Ent	E. coli	FC	HPyVs	HF183
Ent	**Pearson Correlation**	1	.959	.941	.816	.875
	Sig. (2-tailed)		.000	.000	.007	.002
E. coli	**Pearson Correlation**	.959	1	.994	.851	.884
	Sig. (2-tailed)	.000		.000	.004	.002
FC	**Pearson Correlation**	.941	.994	1	.828	.864
	Sig. (2-tailed)	.000	.000		.006	.003
HPyVs	**Pearson Correlation**	.816	.851	.828	1	.980[**]
	Sig. (2-tailed)	.007	.004	.006		.000
HF183	**Pearson Correlation**	.875	.884	.864	.980	1
	Sig. (2-tailed)	.002	.002	.003	.000	

Correlation of qPCR vs. Membrane Filtration Measurements of FIB in Ambient Waters
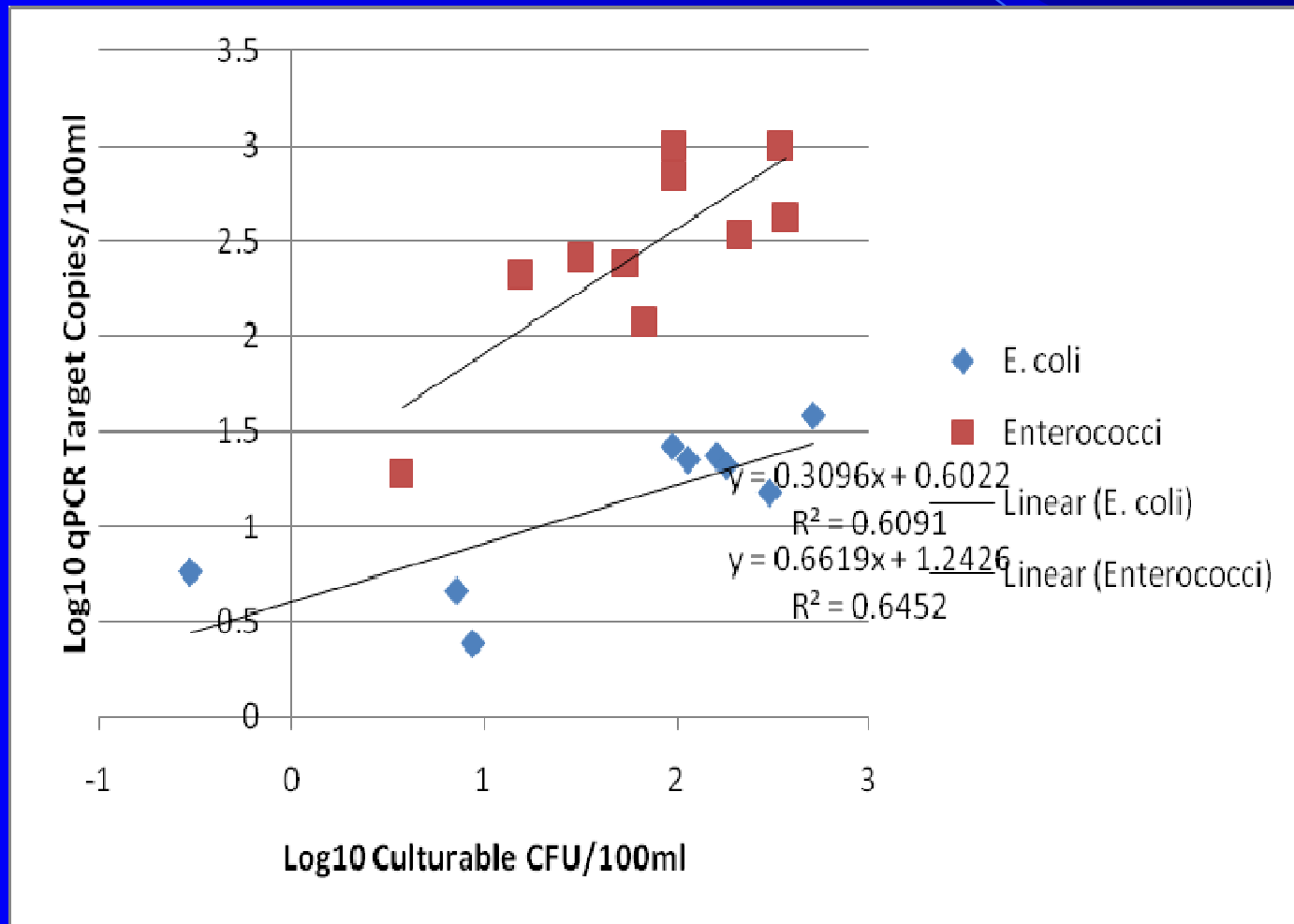
Benefits

- Tools for detecting human source pollution with well-defined performance characteristics
- Quantitative PCR for fecal indicator bacteria can provide more rapid notification of water quality issues
- May eventually be less expensive than culture methods so better sampling coverage can be obtained

Benefits

- **qPCR measurements may provide better correlation with human health outcomes than current culture-dependent methods**

- **Correlation of MST measurements with human health outcomes will inform:**
  - **Management decisions (beach, land)**
  - **Remediation activities**
  - **Risk assessment**

Questions?
vharwood@usf.edu

Table 2-3. Primers and Probes Used for qPCR Assays.

Target	Primer/Probe	Sequence
HPyVs	SM2	5' – AGT CTT TAG GGT CTT CTA CCT TT – 3'
	P6	5' – GGT GCC AAC CTA TGG AAC AG – 3'
	KGJ3	5' – (FAM) TCA TCA CTG GCA AAC AT (MGBNFQ) – 3'
HF183	HF183F	5' – ATCATGAGTTCACATGTCCG – 3'
	SSHBacR	5' – TACCCCGCCTACTATCTAATG – 3'
	SSHBac-PRB	5' – (FAM) TTAAAGGTATTTTCCGGTAGACGATGG (TAMRA) – 3'
E. coli	Eco-F	5' – GTCCAAAGCGGCGATTTG- 3'
	Eco-R	5' – CAGGCCAGAAGTTCTTTTTCCA – 3'
	Eco-PR	5' – (FAM) ACGGCAGAGAAGGTA (MGB NFQ) – 3'
Enterococci	Entero1A (ECST748F)	5' – GAGAAATTCCAAACGAACTTG – 3'
	EnteroR1 (ENC854R)	5' – CAGTGCTCTACCTCCATCATT – 3'
	GPL813TQ	5' – (FAM) TGGTTCTCTCCGAAATAGCTTTAGGGCTA (TAMRA) – 3'
General Bacteroidales	GenBacF3	5' – GGGGTTCTGAGAGGAAGGT – 3'
	GenBacR4	5' – CCGTCATCCTTCACGCTACT – 3'
	GenBacP2	5' – (FAM) CAATATTCCTCACTGCTGCCTCCCGTA (TAMRA) – 3'
IAC	UCP1	5' – (VIC) CCTGCCGTCTCGTGCTCCTCA (TAMRA) – 3'

Anomalous Points on the Standard Curve – Should We Throw Out "Outliers"?

Standard Curve

$y = -3.6723x + 42.677$
$R^2 = 0.9825$